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Citation	Journal of Nanomedicine & Nanotechnology, 2014, v. 5 n. 4, p. 1000224:1-7
Issued Date	2014
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A Self-assembling Nanomaterial Reduces Acute Brain Injury and Enhances Functional Recovery in a Rat Model of Hypertensive Intracerebral Hemorrhage

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Abstract

Intracerebral hemorrhage (ICH) carries a high morbidity and mortality rate. High systolic blood pressure promotes hematoma growth. A self-assembling peptide (SAP) can achieve immediate hemostasis via formation of a SAP nanofiber scaffold (SAPNS). A minimally invasive aspiration of hematoma plus local delivery of SAPNS may lead to decompression of brain tissue and prevent hematoma growth.

In a rat model of renovascular hypertension, experimental ICH was induced by a local injection of bacterial collagenase IV into the left basal ganglia. At 3.5 hours after induction of ICH, stereotactic clot aspiration or sham aspiration was performed manually. Following hematoma aspiration, an intrastriatal injection of 1% SAP, saline or sham injection was performed. Hematoma volume and brain swelling were quantified at 24 hours after ICH. Brain sections were immuno histochemically processed for myeloperoxidase and CD68 to detect the inflammatory infiltration in the perihematomal area. Perihematomal apoptotic cell death was determined using TUNEL staining. Functional recovery was assessed using neurological severity score and modified limb placement test at 1, 3, 7, 10 days after ICH.

The combined treatment with hematoma removal and locally delivered SAPNS decreased hematoma volume, hematoma growth, brain edema, perihematomal inflammatory cell infiltration and apoptosis, as well as improved sensorimotor functional recovery.

Locally delivered SAPNS after hematoma aspiration may prevent hematoma growth, facilitate the repair of ICH-related brain injury and promote functional recovery. Such combined treatment may be effective in patients with hypertensive ICH.

Keywords: *In vivo*; Intracerebral hemorrhage; Nanomaterial; Rat; Hypertension; Self-assembling peptide nanofiber scaffold

Introduction

Intracerebral hemorrhage (ICH) is a more deadly type of stroke, and neurological disability is common among survivors [1]. Hypertension is the main cause of spontaneous ICH. The International Society of Hypertension (ISH) states that high blood pressure (BP; >140/90 mm Hg) occurs in >80% of ICH patients and is independently associated with a poor functional outcome [2,3]. Mechanisms of ICH-related brain injury in hypertensive patients need to be elucidated. Hematoma growth is consistently associated with increased mortality and poor clinical outcome [4-7]. Therefore, stopping hematoma growth is an important treatment strategy to improve the survival rate and outcome after ICH. Systemically delivered hemostatic therapies can stop bleeding, but both systemic and cerebral circulation may be compromised. Therefore, stereotactic minimal invasive aspiration combined with local delivery of a hemostatic material seems promising, especially in the ultra-early phase. In this study, a rat model of hypertensive ICH was used to investigate the effects of combined mechanical removal of hematoma and local delivery of a nanomaterial on brain injury and functional recovery.

RADA16-I (Ac-RADARADARADARADA-CONH₂ R, arginine; A, alanine; D, aspartate) is a self-assembling peptide (SAP). It contains

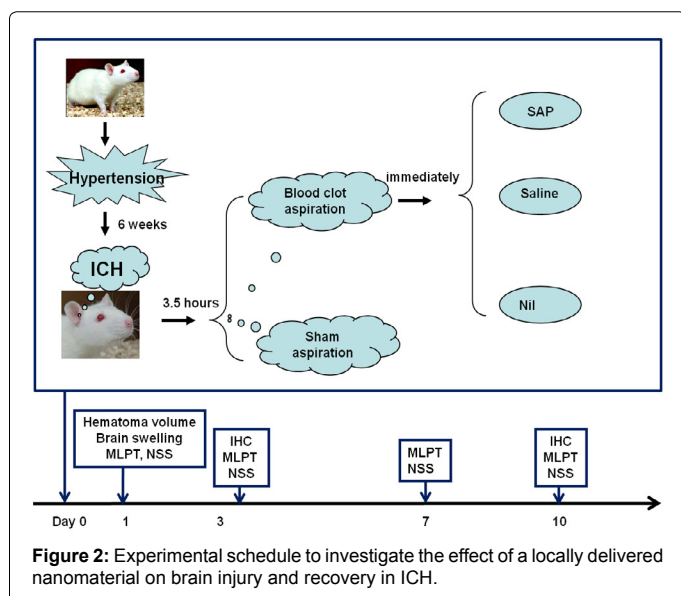
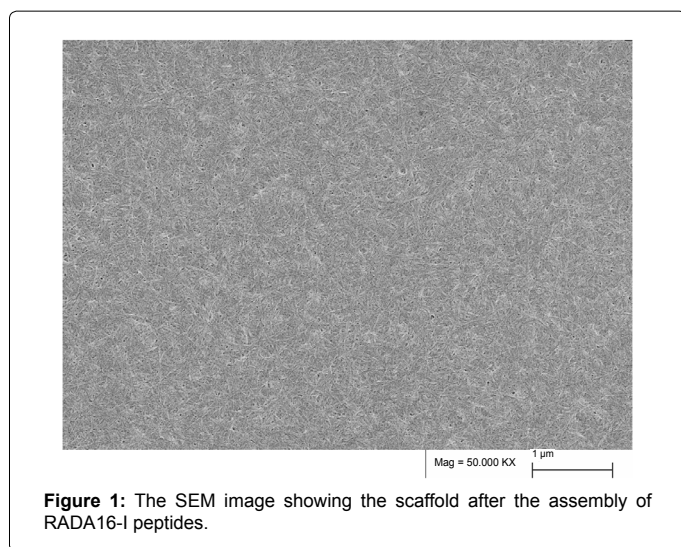
regular repeats of ionic hydrophilic and hydrophobic amino acids which are chemically synthesized and form extremely stable β -sheet structures in water. Thousands of peptides self-assemble to form a single nanofiber, and trillions of peptides or billions of nanofibers form the scaffold that contains \approx 99.5% water and 0.5% peptide materials. The gelation process is charge dependent and accelerated either by changing to a neutral pH or by contacting physiological concentration of salt solutions. The scanning electron microscopy (SEM) image shows the scaffold after the assembly of all of the polypeptides (Figure 1). This peptide can form stable β -sheet structures and eventually turn into a hydrogel [8]. Previous studies in a hamster midbrain severed optical

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Received July 25, 2014; **Accepted** August 20, 2014; **Published** August 27, 2014

Citation: Sang LY, Liang YX, So KF, Leung GK, Ellis-Behnke RG, et al. (2014) A Self-assembling Nanomaterial Reduces Acute Brain Injury and Enhances Functional Recovery in a Rat Model of Hypertensive Intracerebral Hemorrhage. J Nanomed Nanotechnol 5: 224. doi: [10.4172/2157-7439.1000224](http://dx.doi.org/10.4172/2157-7439.1000224)

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tract model have shown that injection of this SAP solution into the incision would lead to regeneration of the optic tract and functional recovery of vision [9]. *In vivo* study has shown that this peptide-based nanofiber scaffold could offer a permissive environment for axon regeneration and accelerate wound healing [8]. Our previous study showed that RADA16-I scaffold could reduce the post-ICH cerebral inflammation, apoptosis, and promote long-term functional recovery in a normotensive animal model [10]. However, it is unknown whether this scaffold would affect ICH-related brain injury in hypertensive animals. Interestingly, this nanomaterial has been found to stop bleeding at surgical sites [11]. It is possible that RADA16-I scaffold can act as a nanobarrier and prevent rebleeding when applied to the lesion cavity after hematoma aspiration.

To establish a hypertensive animal model, the normotensive SD rats were subjected to left renal artery constriction to induce renovascular hypertension (RVHT) [12]. Animals with elevated BP were used to establish an ICH plus ultra-early hematoma aspiration model. Collagenase injection was used to achieve a reproducible hematoma and permit hematoma growth. Stereotactic hematoma aspiration

may result in significant reduction in the hematoma volume and offer a reasonable space for administration of the SAP solution or normal saline. Hematoma volume and brain swelling 24 hours after ICH were assessed, and brain sections were processed for immunohistochemistry (IHC) to assess apoptosis and inflammation. Functional recovery was assessed using modified limb placement test (MLPT) and neurological severity score (NSS). Our findings showed that the RADA16-I scaffold replaced the brain hematoma, prevented hematoma growth, reduced acute brain injury, and improved functional recovery.

Methods

Experimental protocols were approved by the Committee on the Use of Live Animals in Teaching and Research, the University of Hong Kong. Male Sprague-Dawley rats, 6-8 weeks old and weighing 260-280 g, were used in this study. Animals were kept on a 12 / 12 light / dark cycle with *ad libitum* access to food and water. The animal holding areas were under constant monitoring, and the temperature was kept at $23 \pm 2^\circ\text{C}$.

RVHT was induced by applying a silver clip to the left renal artery [13]. Six weeks after renal artery constriction, hypertensive rats were randomly assigned to one of the four groups: the ICH only group, with induction of ICH and sham hematoma aspiration; the aspiration only (Asp) group, with induction of ICH and subsequent hematoma aspiration; the saline group, with induction of ICH, hematoma aspiration, and intralesional injection of saline; and the SAP group, with induction of ICH, hematoma aspiration, and intralesional injection of the SAP solution. The experimental procedures are summarized in Figure 2.

The first cohort of rats ($n = 4-6$ per group, a total of 19 rats) were sacrificed 1 day after ICH for the assessment of hematoma volume and brain swelling. The second cohort of rats ($n = 4$ per group, a total of 16 rats) were sacrificed 3 days after ICH for assessment of perihematomal infiltration of neutrophils and apoptosis. The third cohort of rats ($n = 21$) were sacrificed 10 days after ICH for the evaluation of infiltration of microglia/macrophages and functional assessment. The latter was also performed on day 1, 3, and 7 after ICH.

RVHT model and BP measurement

RVHT was induced by applying a solid silver clip onto the left renal artery (2K1C model), leaving the contralateral artery undisturbed [14,15]. In brief, 6 to 8 week-old male rats were anesthetized with an intraperitoneal injection of ketamine (67 mg/kg) and xylazine (6 mg/kg). After anesthesia, a 3-cm median longitudinal skin incision was made in the abdomen. The left renal artery was stretched and separated from the left renal vein. A silver clip with an inner diameter of 0.25 mm was applied onto the exposed left renal artery as close to the aorta as possible. Systolic BP (SBP) was measured weekly after the unilateral renal artery constriction using an indirect tail-cuff method as described previously [16]. Rats were observed for 10 min daily to assess their general behavior, including grooming and exploratory activities, level of alertness, and physical well-being. Rats with SBP > 150 mmHg at 6 weeks after the renal artery constriction were regarded as RVHT rats and used in subsequent ICH study.

Hematoma aspiration with or without intralesional injection after ICH

Experimental ICH was induced via an intrastriatal injection of type IV collagenase [17,18]. In brief, a burr hole of 2 mm diameter was drilled along the left coronal suture at 3.0 mm lateral to the bregma.

A 30-gauge (G) needle was inserted into the left striatum with its tip at 0.2 mm anterior to the bregma, 3 mm lateral to the midline and 6 mm underneath the dural surface. ICH was induced by a slow injection of 0.12 units collagenase IV (Sigma-Aldrich, St. Louis, USA) in 1.0 μ L saline into the left striatum over 10 min. Three and a half hours after collagenase injection, aspiration was achieved by gentle suction through a 1 mL syringe attached to a 23-G needle. The needle was placed at the same stereotactic coordinates as the collagenase injection. Four aspirations were performed over 15 min. Following aspiration, 20 μ L of 1% RADA16-I solution or saline was injected into the lesion via a 25-G needle. The rate of infusion was 500 μ L per hour. Finally, the burr hole was sealed with bone wax, and the wound was sutured. In the ICH only group, a 23-G needle was placed at the hematoma center for 4 attempts over 15 min, but aspiration was not done.

Hematoma volume and brain swelling

Hematoma volume and brain swelling were quantified 24 hours after ICH. In brief, the rats were transcardially perfused with normal saline followed by 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brain tissue was sliced into eight 1 mm-thick coronal slices using an adult rat brain matrix (World Precision Instruments, Inc., Sarasota, FL). Images of the tissue slices were taken using a digital camera and analyzed using Image J software (NIH, USA) by an observer blinded to group identity. Hematoma volume was calculated by summing the lesion area on each slice and multiplied by the slice thickness. Hemispheric brain swelling after ICH was calculated as the increased volume of ipsilateral hemisphere with respect to the volume of contralateral hemisphere. Data were presented as the percentage increase in brain swelling and expressed as (ipsilateral volume excluding ventricle – contralateral volume excluding ventricle) / contralateral volume \times 100% [19].

Immunohistochemical analyses

The rat was perfused transcardially with ice-cold saline and then 4% paraformaldehyde in 0.1 M PB for 20 min. The brain was post fixed in 4% paraformaldehyde overnight at 4°C before placed in 30% sucrose containing 0.1 M PB for 4 days. Brain sections at 30 μ m were obtained between 1 mm anterior to the bregma and 2 mm posterior to the bregma using a cryostat at -18°C. Brain sections were affixed on Superfrost Plus slides (Menzel-Glaser, Braunschweig, LS, Germany) and air-dried overnight.

After washing, the brain sections were incubated in non-specific blocking solution (10% goat serum) for 1 hour at room temperature. The slides were then incubated with the primary antibody at 4°C overnight. Neutrophils were detected with the rabbit anti-MPO antibody (1:200; Dako, Glostrup, Denmark); microglia/macrophages were identified using mouse anti-ED-1 antibody (1:200; Serotec, Kidlington, UK). After the primary antibody incubation, the sections were washed 3 times in phosphate buffered saline (PBS) for 10 min each time. The sections were incubated with the biotinylated goat anti-rabbit or goat anti-mouse secondary antibody for 2 hours at room temperature and followed by washing in PBS. To amplify the signals, an avidin-biotin complex solution (1:200; Vector, Burlingame, USA) was used. After 30 min, sections were washed in PBS, and the signal was visualized by 3, 3'-diaminobenzidine (DAB; Vector, Burlingame, CA, USA) for detection of the peroxidase signal. Sections were then cover slipped for examination under a light microscope.

Apoptosis was assessed by terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay with an *in situ* cell death detection kit (Roche, Indianapolis, IN) [20-23]. The fixed brain sections

were blocked with 3% H₂O₂ in methanol to quench the endogenous peroxidase. The sections were incubated in the permeabilization solution and then incubated in labeling reaction mixture for 60 min at 37°C in the dark. After incubating in converter-POD for 30 min at 37°C, sections were stained with DAB-H₂O₂. The slides were examined under a light microscope.

The cell quantification process was conducted in a blinded manner. The number of positive cells in the perihematomal area was counted. The total number of positive cells within these brain sections was converted to cell density to facilitate comparison among the groups.

Functional deficits

Functional deficits were assessed on day 1, 3, 7, and 10 after ICH. The rats were subjected to MLPT and NSS by a blinded observer [24]. MLPT consists of 3 limb-placement tasks that assess the sensorimotor integration of the forelimb and the hind limb by checking responses to tactile and proprioceptive stimulation [24]. First, the rat was suspended at 10 cm above a table, and the stretch of the forelimbs toward the table was scored as follows: 0 point for normal stretch and 1 point for abnormal flexion. Next, the rat was positioned along the table edge, and its forelimbs were suspended over the edge and allowed to move freely. Each limb (forelimb, second task; hind limb, third task) was pulled down gently, and the retrieval and placement were checked. Finally, the rat was placed toward the table edge to check for lateral placement of the forelimbs. The last three tasks were scored in the following manner: 0 point for normal performance, 1 point for performance with a delay (2 seconds) or incomplete performance, and 2 points for no performance. A score of seven points indicates maximal neurological deficit, and a score of 0 point indicates normal performance.

Modified NSS was adopted from previous reports with some modification [25,26]. NSS is a composite of motor, sensory, reflex, and balance tests. Points were awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher score, the more severe is the injury [10]. All animals were trained to be familiar with the testing environment before ICH surgery.

Statistical analysis

Statistical analyses were performed using the Graph Pad Prism (version 5, San Diego, CA, USA). Group differences were assessed by ANOVA followed by LSD *post-hoc* test. For the longitudinal functional assessment, two-way analysis of variance was used. Kruskal-Wallis ANOVA on ranks followed by Mann-Whitney U *post hoc* test was used for data not in normal distribution. Statistical difference was inferred by a P value less than 0.05. Values were in mean \pm SD.

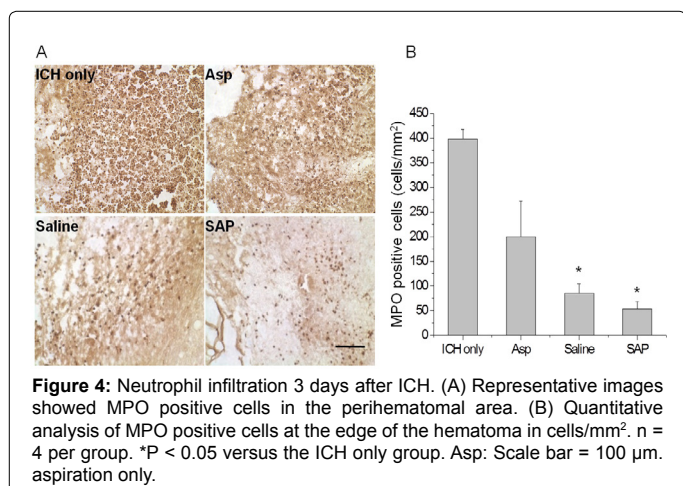
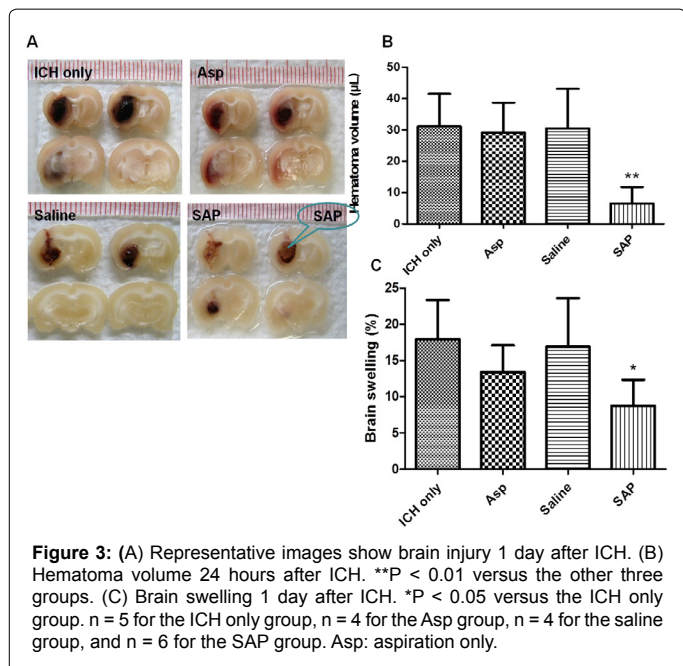
Results

Hematoma volume

In RVHT rats, the hematoma volume was not significantly reduced by aspiration (Figure 3). A significant reduction of hematoma volume was found in the SAP group (6.57 \pm 5.19 μ L) when compared to the other groups (Figure 3). There were no significant differences in hematoma volume among the ICH only group (32.11 \pm 11.70 μ L), the Asp group (29.19 \pm 9.53 μ L) and the saline group (30.49 \pm 12.63 μ L).

Brain swelling

Ipsilateral hemisphere showed greater brain swelling than the contralateral hemisphere 24 hours after ICH in all groups (Figure 3C). However, brain swelling was significantly reduced in the ipsilateral hemisphere of the SAP group (8.80 \pm 3.55%) relative to that in ICH only



group (17.96 ± 5.39%, Figure 3C). The SAP group also demonstrated a trend of less brain swelling than the Asp group and the saline group, but statistical significance was not reached. Brain swelling in the Asp group (13.40 ± 3.71%) and the saline group (16.90 ± 6.71%) was not different from that in the ICH only group.

Cerebral inflammation

MPO immunoreactivity revealed a high density of positive cells within the hematoma and in the perihematoma area in the ICH only group (394.6 ± 24.2 cells per mm²) 3 days after ICH (Figure 4A). However, aspiration only (195.4 ± 68.5 cells per mm²) did not significantly reduce the number of MPO immuno-positive cells. Additional local injection of saline (72.2 ± 23.6 cells per mm²) or SAPNS (53.8 ± 19.16 cells per mm²) significantly reduced the neutrophil infiltration versus the ICH only group (Figure 4B). Moreover, the SAP group tended to further reduce the MPO immune-positive neutrophil counts in perihematoma area.

ED-1 immuno-positive cells had an amoeboid or round phagocytic

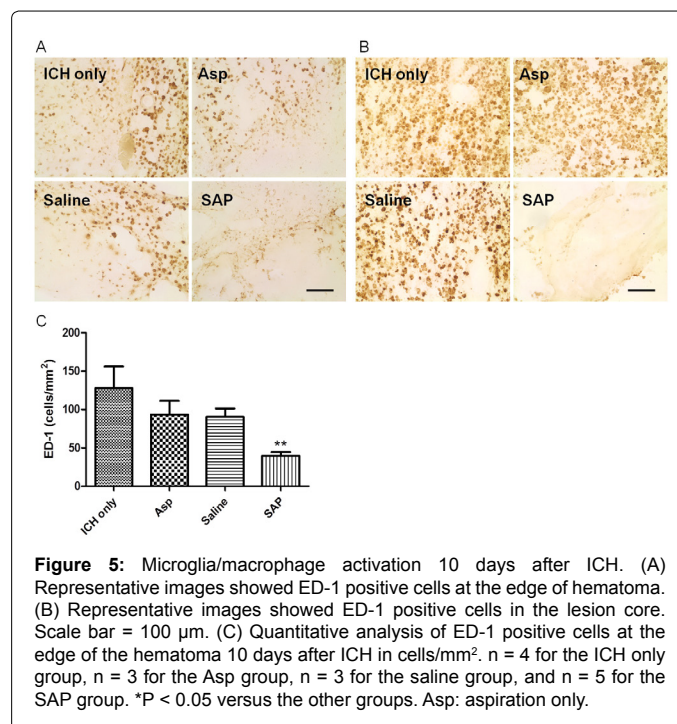
morphology and were found in the perihematoma area and within the hematoma in all groups 10 days after ICH (Figure 5). The perihematoma ED-1 immuno-positive cell densities were similar among the ICH only group (127.8 ± 28.1 cells per mm²), the Asp group (93.6 ± 17.6 cells per mm²) and the saline group (90.3 ± 11.2 cells per mm²). Treatment with SAPNS reduced the number of ED-1 immuno-positive cells (40.1 ± 5.0 cells per mm²) when compared to other three groups (Figure 5C). In the lesion core, there were very few ED-1 immuno-positive cells infiltrating the SAPNS in the SAP group, while there was a high density of ED-1 immuno-positive cells in the ICH only group, the Asp group and the saline group (Figure 5B).

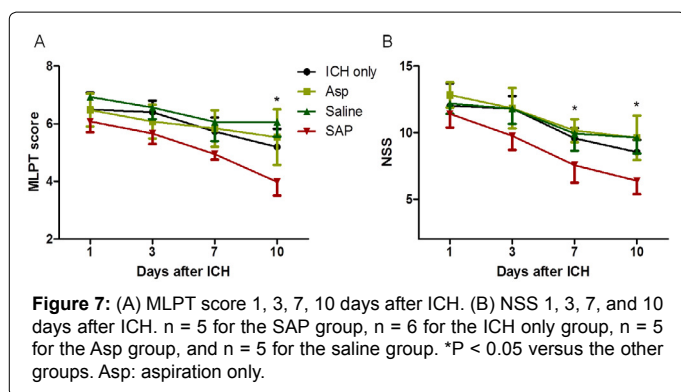
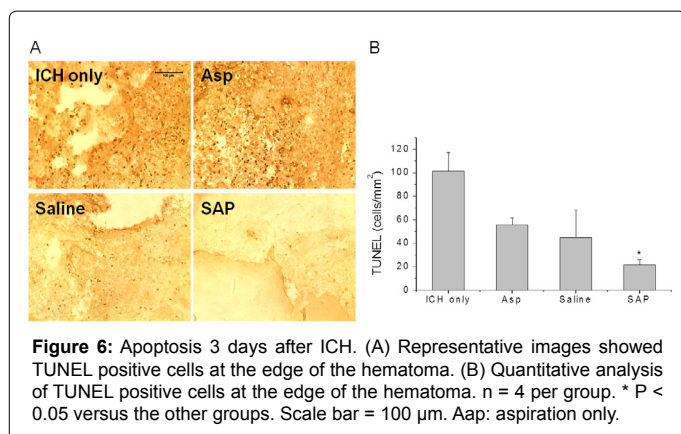
Apoptosis

Three days after ICH, TUNEL revealed a high density of positively labeled cells within the hematoma and in the perihematoma area in the ICH only group (100.9 ± 15.1 cells per mm²; Figure 6). The results were similar in the Asp group (55.5 ± 5.4 cells per mm²) and the saline group (44.4 ± 19.8 cells per mm²; Figure 6) and not significantly different from the ICH only group (Figure 6B). Treatment with the SAP reduced the number of TUNEL-labeled cells (21.2 ± 3.8 cells per mm²) when compared to the other groups.

Functional deficits

All rats survived after the surgery and completed the behavioral tests. MLPT deficits were maximal at 1 week after ICH and showed a trend of recovery within the first 10 days. A significant sensorimotor functional improvement (4.0 ± 0.5) was detected in the SAP group 10 days after ICH (Figure 7A) when compared to the other three groups, viz. the ICH only (5.4 ± 0.5), the Asp group (5.5 ± 1.0), and the saline group (6.1 ± 0.5). According to the NSS, the SAP group exhibited a slightly better performance than the other groups 3 days after ICH (Figure 7B), and the difference became statistically significant 7 days (the SAP group, 7.6 ± 1.3; the ICH only, 9.7 ± 0.8; the Asp, 10.2 ± 0.9; and the saline group, 9.9 ± 1.3) and 10 days after ICH (the SAP group, 6.4 ± 1.0; the ICH only, 8.8 ± 0.7; the Asp, 9.6 ± 1.7; and the saline





group, 9.6 ± 1.2).

Discussion

In this study, combining minimally invasive hematoma aspiration with local delivery of SAPNS attenuated acute brain injury and enhanced functional recovery in a rat model of hypertensive ICH. These effects were associated with the prevention of hematoma growth as well as reduction of brain edema, perihematomal apoptosis and inflammatory reaction.

Hypertension is the single most important risk factor for spontaneous ICH [27-31]. Our previous study showed the SBP of the RVHT rats increased gradually and became stable around levels of 160-190 mmHg at 6 weeks after unilateral renal artery constriction [12]. No spontaneous stroke was seen in the RVHT rats within the study period. This rat model of hypertensive ICH closely mimics ICH patient with elevated BP. Using this model, the present study evaluated the effects of a hemostatic nanomaterial on the ICH-induced brain injury. In consistent with previous studies on open surgical sites [11], local delivery of SAPNS prevented the hematoma growth in the deep brain in the hypertensive rats.

RADA16-I peptide, the SAP used in this study, is composed of repeated sequence of arginine, alanine, aspartate and alanine. A unique characteristic of the RADA16-I is its ability to self-assemble into a nanofiber scaffold upon contact with cations [32]. It can fit into an irregular space and assembly into a scaffold within seconds. Our previous study showed that in normotensive rats, SAPNS replaced deep brain hematoma, reduced the brain cavity formation [ref. in press]. The SAP has also been shown to achieve hemostasis when applied to open bleeding sites [11]. Our previous study showed that

hypertension aggravated ICH-related brain injury and deficits in the rat [12]. Moreover, stereotactic aspiration of blood led to decompression of brain tissue in these normotensive rats [13]. In the present study, the RADA16-I SAP solution could fill in brain cavity created by hematoma aspiration and, more importantly, it could stop hematoma growth in the hypertensive rats. The present results revealed for the first time that intracerebral administration of SAP solution following hematoma aspiration during the hyperacute stage of ICH exhibited hemostatic effect. In addition, the building blocks of RADA16-I are made of pure natural L-amino acids. The amino acids generated from degradation of RADA16-I can be utilized as an additional source of amino acids, and toxic effects of the SAP solution was not seen in various animal models [8,9,33].

Brain edema and inflammation are important mechanisms underlying ICH-associated brain injury [9,21,26,34]. Brain swelling due to edema is a marker for tissue damage [35] and a key contributor to raised intracranial pressure (ICP) [36]. High ICP reduces cerebral perfusion pressure [37] and causes brain ischemia, herniation, and/or brain death [35,37,38]. Blood breakdown products may contain high concentrations of detrimental factors, such as iron and thrombin [39]. Free iron may mount a free radical-mediated damage of the endothelium and aggravate blood-brain-barrier (BBB) dysfunction [40,41]. Reduction of iron release from lysed red blood cells is associated with reduced brain edema [42]. Thrombin mediates a detrimental role via activation of the inflammatory cascade [43-48]. Firstly, it activates the inflammatory cascade via chemotaxis of leukocytes, expression of adhesion molecules, and release of inflammatory cytokines. Secondly, thrombin enhances expression of matrix metalloproteinases, which can break down BBB and cause edema formation [49]. Inflammation and brain edema were reduced in the SAP treatment group. Possible explanations include the followings. Decreased hematoma volume is associated with reduced amount of toxic blood breakdown products and less inflammatory cytokines/chemotactic factors. In addition, these detrimental mediators may diffuse into the SAPNS and therefore their concentrations in the perihematomal area are reduced.

Although ICH patients have a high mortality rate of 31% 7 days after onset [50,51], the mortality rate was zero within 10 days after experimental ICH in the hypertensive rats in this study because we used a low dose of collagenase IV (0.12 U) to induce the ICH. Intrastriatal injection of 0.12 U of collagenase IV in RVHT rats would produce a relative small hematoma not extending to the lateral ventricle, and the aspiration procedure was successful without noting CSF in the aspirated liquid. Despite zero mortality rate, severe sensorimotor deficits were seen in all the groups of hypertensive rats. The reduced hematoma volume in the SAP treatment group could have limited the secondary brain damage and accounted for the improved functional recovery. Future studies may be conducted to examine the survival of surrounding neuronal cells, endogenous neurogenesis and repair of brain circuits within and around the lesion site.

In conclusion, we applied a nanomaterial into the deep brain cavity immediately after hematoma aspiration in a rat model of hypertensive ICH. Our results suggest that the SAP can serve as a biocompatible material, reduce acute brain injury, and improve functional recovery. The present study therefore provides some evidence for the potential application of SAPNS in patients with hypertensive ICH.

Acknowledgement

This work was supported by the General Research Fund of the Research

Grants Council (HKU763109M), the Committee on Research and Conference Grants (201007176091) and the Stanley Ho Alumni Challenge Funds (208040030, 207051026).

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Citation: Sang LY, Liang YX, So KF, Leung GK, Ellis-Behnke RG, et al. (2014) A Self-assembling Nanomaterial Reduces Acute Brain Injury and Enhances Functional Recovery in a Rat Model of Hypertensive Intracerebral Hemorrhage. *J Nanomed Nanotechnol* 5: 224. doi: [10.4172/2157-7439.1000224](https://doi.org/10.4172/2157-7439.1000224)

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